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Conditionally Replicating Plasmid Vectors That Can Integrate into the *Klebsiella pneumoniae* Chromosome via Bacteriophage P4 Site-Specific Recombination

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P4 is a satellite phage of P2 and is dependent on phage P2 gene products for virion assembly and cell lysis. Previously, we showed that a virulent mutant of phage P4 (P4 *vir*1) could be used as a multicopy, autonomously replicating plasmid vector in *Escherichia coli* and *Klebsiella pneumoniae* in the absence of the P2 helper. In addition to establishing lysogeny as a self-replicating plasmid, it has been shown that P4 can also lysogenize *E. coli* via site-specific integration into the host chromosome. In this study, we show that P4 also integrates into the *K. pneumoniae* chromosome at a specific site. In contrast to that in *E. coli*, however, site-specific integration in *K. pneumoniae* does not require the *int* gene of P4. We utilized the alternative modes of P4 lysogenization (plasmid replication or integration) to construct cloning vectors derived from P4 *vir*1 that could exist in either lysogenic mode, depending on the host strain used. These vectors carry an amber mutation in the DNA primase gene α, which blocks DNA replication in an Su^- host and allows the selection of lysogenic strains with integrated prophages. In contrast, these vectors can be propagated as plasmids in an Su^+ host where replication is allowed. To demonstrate the utility of this type of vector, we show that certain nitrogen fixation (*nif*) genes of *K. pneumoniae*, which otherwise inhibit *nif* gene expression when present on multicopy plasmids, do not exhibit inhibitory effects when introduced as merodiploids via P4 site-specific integration.

P4 is a small satellite phage of the P2 family of phages (27); its lytic cycle requires morphological and lysis gene products provided by a helper phage such as P2 (26). Using P2-coded proteins, P4 directs the assembly of virions morphologically identical to those of its helper, except for capsids that are only one-third of the normal volume (15). The smaller size capsid accommodates the 7.5×10^6-dalton P4 chromosome (15) but excludes the larger 22×10^6-dalton chromosome of P2 (10, 14). In the absence of a P2 helper, wild-type P4 has been shown to lysogenize by phage-specific integration into the *Escherichia coli* chromosome at about 96 min (8, 27). In this paper, we report that phage P4 can also integrate into the *Klebsiella pneumoniae* chromosome via site-specific integration, but unlike the case in *E. coli* (8), integration into *K. pneumoniae* does not require the P4-encoded *int* gene.

A second route of lysogenization for certain P4 mutants, exemplified by immunity-insensitive P4 *vir*1, is plasmid formation in *E. coli* and *K. pneumoniae* (11, 21). In a previous publication, we took advantage of the ability of P4 *vir*1 to replicate as a plasmid to construct P4 "phasmid" (phage-plasmid) cloning vectors which could exist either as a high-copy-number autonomously replicating plasmid (in the absence of a P2 helper) or as a packaged phage particle. In this study, we have taken advantage of the ability of P4 to integrate site specifically to construct P4 cloning vectors, which, due to the presence of a nonsense (chain termination) mutation in the essential P4 primase gene (4), can either integrate into the host chromosome or replicate as a multicopy plasmid, depending on the presence of a tRNA nonsense-codon suppressor in the host strain. When these vectors are used, DNA fragments can be cloned and propagated in a multicopy plasmid state. Subsequently, a single copy of the plasmid can be integrated into the chromosome for physiological studies. As an example of how these vectors can be used, we show that certain *nif* DNA fragments of *K. pneumoniae*, which inhibit nitrogen fixation when present on a high-copy-number plasmid (6, 23), do not exhibit this inhibitory phenotype when integrated into the

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K. pneumoniae chromosome in a single-copy state.

MATERIALS AND METHODS

Media. Rich media (LB and LC) and nitrogen-deficient medium (NFDM) have been described previously (23, 24). Tetracycline and kanamycin, when necessary, were used at 10 and 20 μg/ml, respectively. Previously reported procedures in which LC medium was used for the preparation of high-titer P2 and P4 phage stocks were followed with hosts C1792 and DO25, respectively (1).

Assays. Acetylene reduction assays for nitrogenase activity were performed according to Riedel et al. (23).

DNA biochemistry. The rapid alkaline lysis procedure (16) for small-scale plasmid preparations was used throughout for analyses of recombinant P4 molecules. Enzymes used in recombinant DNA techniques were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used according to the specifications of the manufacturer.

Strains and strain construction. Bacterial, bacteriophage, and plasmid strains are listed in Table 1.

Construction of P4DO100. A recombinant plasmid was constructed by ligating the 8.1-kilobase-pair (kb) *Eco*RI fragment of P4 *vir*1 *sid*1 into the *Eco*RI site of pHC79 (13). This plasmid (pDO5) was partially cleaved with *Eco*RI and subjected to limited digestion by exonuclease *Bal* 31, thus eliminating the exposed *Eco*RI ends. After digestion with endonuclease *Bgl*II and DNA polymerase I (large fragment) in the presence of dXTPs, the linear molecules were recircularized in the presence of T4 ligase. Intermolecular joining of a DNA polymerase I-filled *Bgl*II end and a *Bal* 31-treated blunt end regenerated a *Bgl*II site in one out of every four clones. P4DO100 is one such plasmid with both a single *Eco*RI site and a single *Bgl*II site. The structure of P4DO100 is shown in Fig. 1B.

Construction of P4DO102. To select for the minority of phage that integrate among the vast majority of phage that replicate as high-copy plasmids, the αam52 allele was crossed into P4DO100. The *sid*1 mutation permits recombinant molecules in the range of 10 to 17 and 23 to 31 kb to grow lytically in the presence of helper phage P2 (D. Ow, unpublished data). Due to the lack of a tight selection, the *sid*$^+$ allele was crossed in along with the αam mutation; as a result, P4DO102 is *vir*1 αam52, but *sid*$^+$. The structure of P4DO102 is identical to that of P4DO100.

Construction of P4DO104. P4DO104 was derived by replacing the Tc^r gene of P4DO102 with a Km^r fragment from Tn*903*. This was accomplished by first inserting a *Bam*HI Km^r fragment (made by ligating

TABLE 1. Strain list

Strain	Genotype	Relevant properties	Source
E. coli			
C-436	*arg*(Am) T1^r *trp his rpsL*	Su$^-$	31
C-1757	*arg*(Am) T1^r *trp supD rpsL*	Su$^+$	31
C-1792	*arg*(Am) T1^r *trp*(Am) *his supF rpsL*	Su$^+$	31
HB101	*pro leu thi lacY hsdR hsdM endA recA rpsL20 ara-14 galK2 xgl-5 mtl-1 supE44*	Su$^+$	5
DO25	C-1792(P2 *A*am127)	Su$^+$, P2 lysogen	This paper
CK111	*recA13*	Rec$^-$, Su$^-$	8
K. pneumoniae			
M5a1	Prototrophic	Wild type	S. Streicher
KP5014	*hspR*	Su$^-$	21
KP5614	*hisD hspR recA56 srl*	Rec$^-$, Su$^-$	21
KP5617	*hisD hspR recA56 srl nifB*	Rec$^-$, Su$^-$, NifB$^-$	This laboratory
KP5611	*hisD recA56 srl nifA*	Rec$^-$, Su$^-$, NifA$^-$	This laboratory
UN4102	*hisD lacZ recA56 srl*::Tn*10* Gal^r	Rec$^-$, Su$^-$, LacZ$^-$	20
Phages and plasmids			
P2 *A*am127	*A*am127	Replication defective	18
P4 *vir*1	*vir*1	Immunity insensitive	19
P4 *vir*1 αam52	*vir*1 αam52	Immunity insensitive, replication defective	1
P4 *vir*1 *sid*1	*vir*1 *sid*1	Immunity insensitive, large capsids	25
P4 *c*I405	*c*I405	Defective in the establishment of prophage	8
P4DO100	*vir*1 *sid*1 Tc^r	Replication proficient, Tc^r	This paper
P4DO102	*vir*1 αam52 Tc^r	Replication defective, Tc^r	This paper
P4DO104	*vir*1 αam52 Km^r	Replication defective, Km^r	This paper
P4DO105	*vir*1 αam52 Tc^r Km^r	Replication defective, Tc^r, Km^r	This paper
pDO201	*vir*1 αam52 Km^r *nifLABQ*	*nifLABQ*, Km^r	This paper
pVSA3	*vir*1 αam52 Km^r *nifH-lacZ* fusion	*nifH-lacZ* fusion	30

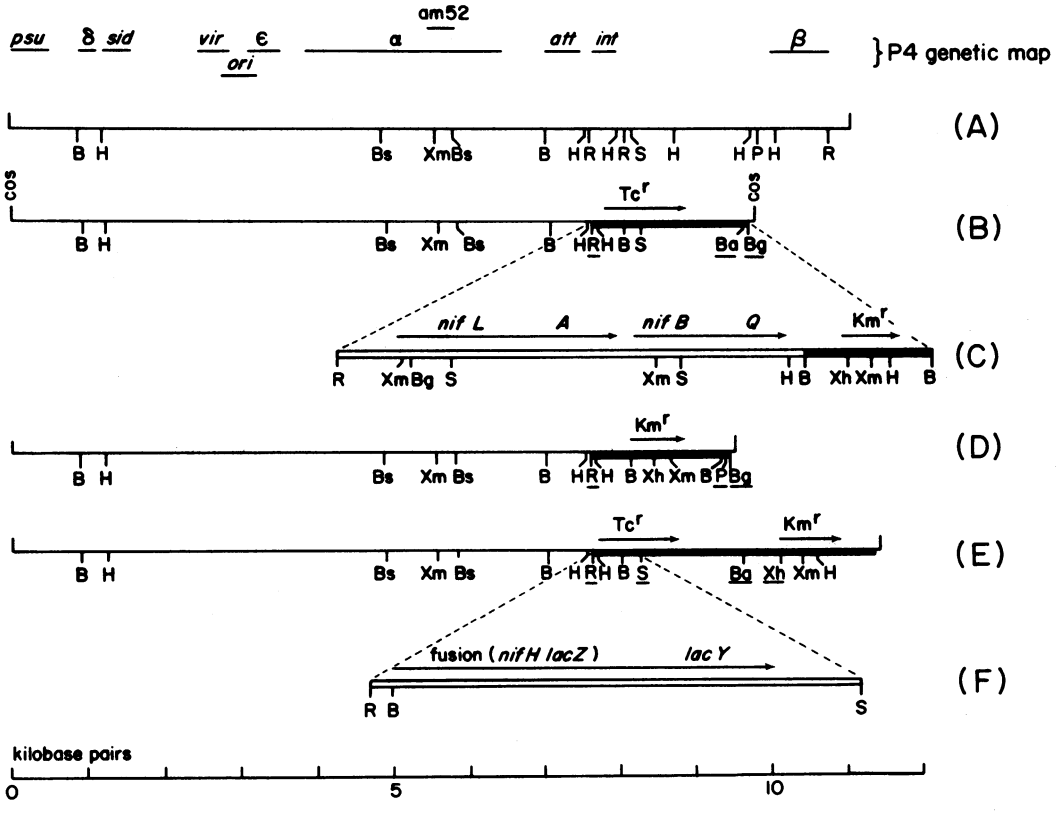

FIG. 1. Physical and genetic maps of (A) P4, (B) P4DO100 and P4DO102, (B plus C) pDO201, (D) P4DO104, (E) P4DO105, and (E plus F) pVSA3, as linearized at the P4 cohesive ends (*cos*). pDO201 is the DNA fragment shown (C) cloned into P4DO102; pVSA3 is the DNA fragment shown (F) cloned into P4DO105. Only selected endonuclease sites are shown: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Xh, *Xho*I; Xm, *Xma*I (*Sma*I). An underlined site indicates that it is a unique site which, when used for cloning, leaves at least one antibiotic resistance phenotype intact. For more detailed endonuclease restriction maps of fragments derived from P4, pHC79, and Tn*903*, refer, respectively, to references 17, 13, and 12. A brief description of P4 genes is included in reference 7.

*Bam*HI molecular linkers to the ends of the *Pvu*II fragment of Tn*903*) into the *Bam*HI site of a "polylinker" (*Eco*RI-*Bam*HI-*Pst*I-*Bgl*II) present on the plasmid πVX (from B. Seed). Subsequently, the Km^r gene was excised as an *Eco*RI-*Bgl*II fragment and used to replace the *Eco*RI-*Bgl*II Tc^r gene of P4DO102. The resultant recombinant is the Km^r phage P4DO104. The structure is shown in Fig. 1D.

Construction of P4DO105. P4DO105 was constructed by inserting the *Bam*HI Km^r fragment (derived from Tn*903*) into the *Bgl*II site of P4DO102. Thus, it is both Tc^r and Km^r. The structure is shown in Fig. 1E.

Construction of pDO201. pDO201 was constructed by ligating together three purified restriction fragments: (i) the *Eco*RI-*Bgl*II 8.1-kb vector fragment of P4DO102, (ii) the *Eco*RI-*Bam*HI fragment encoding *nifLABQ* from pGR116 (19), and (iii) a *Bam*HI fragment encoding the Km^r gene of Tn*903*. The structure is shown in Fig. 1B plus C.

pVSA3 has been described previously (30). It was constructed by replacing the *Eco*RI-*Sal*I fragment of P4DO105 with an *Eco*RI-*Sal*I fragment (encoding the *nifH-lacZ* protein fusion) from the pBR322-derived plasmid pVSA2. The structure is shown in Fig. 1E plus F.

Transduction. Cells were grown in LC broth to a density of 2×10^8 cells per ml and infected with phage at a multiplicity of five phage per cell (Table 2). After 5 min of incubation on ice, each infected culture was diluted twofold with LB broth and placed standing in a 37°C water bath. After 1 h of incubation at 37°C, all cultures were serially diluted and titers were determined for colonies on permissive medium (LB) and selective medium (LB medium with 10 μg of tetracycline per ml).

RESULTS

Construction of a prototype vector. In an earlier study, we reported that the 8.1-kb *Eco*RI fragment of phage P4 *vir*1 can replicate autonomously as a high-copy plasmid (21) (in Fig. 1, P4 molecules are drawn as linearized at the cohesive ends [*cos*]; consequently, the 8.1-kb *Eco*RI fragment of P4 is shown as two fragments dis-

TABLE 2. Lysogenization frequencies of P4DO102[a]

Infecting phage	Lysogenization frequency with following host strain:					
	E. coli					*K. pneumoniae*
	C-1757 (*supD*)[b]	C-1792 (*supF*)	HB101 (*supE*)	C-436 (Su^-)	CK111 (Su^- Rec^-)	KP5614 (Su^- Rec^-)
Uninfected control	$<3 \times 10^{-8}$	$<7 \times 10^{-8}$	$<8 \times 10^{-8}$	$<5 \times 10^{-8}$	$<5 \times 10^{-8}$	$<1 \times 10^{-7}$
P4DO100			0.68	0.70	0.81	0.78
P4DO102	0.29	0.62	0.54	$<4 \times 10^{-8}$	3×10^{-7}	2×10^{-3}[c]
P4 *c*I405				$<4 \times 10^{-8}$	$<1 \times 10^{-5}$	$<1 \times 10^{-7}$
P4DO102 + P4 *c*I405				2×10^{-3}	0.31	1×10^{-2}[c]
P4 *vir*1 αam52				$<5 \times 10^{-8}$	$<3 \times 10^{-8}$	$<1 \times 10^{-7}$
P4DO102 + P4 *vir*1 αam52				6×10^{-6}	0.44	1×10^{-2}[c]
P2 *A*am127						$<1 \times 10^{-7}$
P4DO102 + P2 *A*am127						4×10^{-4}[c]

[a] Phage infections were performed and lysogenization frequencies were determined as described in the text. A blank space indicates that values were not determined.

[b] Relevant properties are indicated within parentheses.

[c] Two classes of Tc^r transductants were observed on plates with 10 μg of tetracycline per ml. Approximately 10% of the total transductants grew into normal-size colonies within 24 h. The remaining majority of transductants exhibited a slower-growing phenotype and formed normal-size colonies only after 48 h of incubation.

connected at the *cos* site). Because this 8.1-kb fragment also contains all of the essential genes for P2 helper-dependent growth (29), we showed that it could be used as a phasmid cloning vector (21). Furthermore, we demonstrated that the establishment of a multicopy plasmid state is the predominant form of lysogenization of the 8.1-kb fragment because an integrated form could not be detected in the *K. pneumoniae* chromosome by DNA hybridization. Subsequently, Calendar et al. (8) found that in *E. coli*, integration requires both the P4 *att* site located at the end of the 8.1-kb *Eco*RI fragment and a P4-encoded *int* gene product present on an *Eco*RI fragment adjacent to the 8.1-kb fragment.

On the basis of the above findings, we hypothesized that it should be possible to construct cloning vectors derived from P4 *vir*1 that could either integrate site specifically or enter into a multicopy plasmid mode of replication. In theory, the same 8.1-kb *Eco*RI fragment used earlier as a phasmid vector could also be used as the basis of an integrating vector (i) if DNA replication could be conditionally suppressed and (ii) if the *int* function could be provided in trans. To test this hypothesis, we constructed P4DO102, a derivative of P4 *vir*1 composed of two DNA fragments. One is the 8.1-kb *Eco*RI fragment of P4 *vir*1 which contains genes necessary for P2 helper-dependent lytic growth, P2 helper-independent DNA replication, the site-specific recombination locus *att*, and an amber allele (am52) of the DNA primase gene α (4). The other fragment in P4DO102 is a 2-kb *Eco*RI-to-*Bgl*II fragment which contains genes for tetracycline resistance and which was derived from the pBR322 derivative pHC79 (13). P4DO102 contains unique recognition sites for endonucleases *Eco*RI, *Bgl*II, and *Bal*I and can be packaged into P4 phage heads. A detailed description of the construction of P4DO102 (and its replication-proficient α^+ parent, P4DO100) is described above.

The plasmid copy number of P4DO102 is lower than that of its parent, P4DO100, in *supD*, *supE*, and *supF* strains thus far examined. The yield of P4DO102 plasmid DNA from any of these Su^+ strains resembles that of strains harboring pRK248, a plasmid that has a copy number of ca. six to eight (32). In contrast, the yield of P4DO100 plasmid DNA from either a Su^+ or a Su^- host resembles that of strains harboring multicopy plasmids such as pACYC184 (9). We suspect that the lower copy number of P4DO102, compared with P4DO100, is due to poor suppression of the αam52 mutation. Because of the Tc^r phenotype conferred by P4DO102, we were able to assay conveniently for either integration or plasmid formation in various host backgrounds. As expected, P4DO102 efficiently transduced a Tc^r phenotype after infection of a *supD*, *supE*, or *supF E. coli* host, but could not confer Tc^r to *sup*^{0} (Su^-) *E. coli* strains, presumably due to its inability to form plasmids or integrate (Table 2). In contrast, P4DO100, the replication-proficient (α^+) parent of P4DO102, was capable of transducing the *sup*^{0} strains to Tc^r.

We next examined the ability of P4DO102 to integrate into the *E. coli* chromosome when the *int* gene was provided in trans. P4 *c*I405 is a P4 clear-plaque mutant which by itself cannot integrate but can provide the *int* function in trans to promote integration for an otherwise *int*$^-$ *att*$^+$ P4 phage (8). Consistent with previous results, P4 *c*I405 complemented P4DO102 for lysogenization in *recA E. coli* CK111 (Table 2). Approximately one-third of the infected CK111 cells became Tc^r after coinfection of P4DO102 and P4 *c*I405 each at a multiplicity of infection of five particles per cell. Similar frequencies were obtained when P4 *vir*1 αam52, which contains the same α mutant allele as P4DO102, was used as the *int*$^+$ helper. Although the presence of plasmids was not examined in these two cases, site-specific integration was implicated because P4 *vir*1 αam52 (which can neither complement nor marker rescue the amber mutation in P4DO102) promoted the same high frequency of lysogenization as did P4 *c*I405.

In contrast to the results obtained with strain CK111, when another *E. coli* strain (C-436) was used as the host for coinfection with P4DO102 and P4 *c*I405, the frequency of Tc^r C-436 cells obtained was two orders of magnitude lower than with CK111 (Table 2). When P4 *vir*1 αam52 was used as a helper, a very low frequency (6×10^{-6}) of infected C-436 cells acquired Tc^r. In general, we found a great deal of variability among *E. coli* strains in their ability to act as recipients for P4DO102 site-specific integration (data not shown). We have made no attempt to distinguish whether this variation in integration efficiency among strains is due to the presence (or absence) of the preferred bacterial *att* locus, sequence divergence of the bacterial *att* region, or differential expression or activity or both of a host factor(s) involved in P4-specific recombination.

P4-encoded *int* gene not required for P4 site-specific integration into the *K. pneumoniae* chromosome. We were particularly interested in determining whether P4DO102 could be complemented for site-specific integration in *K. pneumoniae*. As mentioned earlier, certain loci (at least four) in the *K. pneumoniae* nitrogen-fixation (*nif*) regulon inhibit N_2 fixation when cloned into high-copy plasmid vectors and introduced into a wild-type strain (6, 23). It is likely that *nif* inhibition is due to the titration of a limited amount of a *nif*-specific regulatory factor which is required for the activation of several *nif* operons. The development of an integrating vector for *K. pneumoniae* would overcome the high-copy inhibitory effects of certain *nif* sequences, and it would also provide a more natural physiological state (as opposed to multicopy plasmids) to study gene regulation.

As in the case of *E. coli* CK111, coinfection of P4DO102 with either P4 *c*I405 or P4 *vir*1 αam52 into several *K. pneumoniae* strains yielded Tc^r transductants (Table 2) at a rate of ca. 0.1%, a frequency several orders of magnitude above that of the reversion rate of the αam52 allele. Anomalously, however, a similar result was obtained when P4DO102 was used in an infection without a helper. This is not a strain-specific phenomenon, since several other derivatives of wild-type *K. pneumoniae* M5al (KP5014, KP5617) behaved similarly (data not shown). We also noticed that only 10% of the Tc^r transductants formed colonies within 24 h. The majority (about 90%) of the Tc^r transductants exhibited a slower-growing phenotype on plates containing 10 μg of tetracycline per ml and formed colonies only after 48 h of incubation. (These two classes of transductants may be correlated with a dosage effect of the Tc^r gene [see below].)

There are several reasonable explanations for the fact that P4DO102 can transduce *K. pneumoniae* Su$^-$ strains. One possibility is that *K. pneumoniae* has a gene equivalent to the P4 DNA primase gene which permits P4DO102 to replicate autonomously. This explanation is unlikely, however, because P4DO102 cannot form plaques on a Su$^-$ *K. pneumoniae*(P2) lawn. Furthermore, none of the 24 Tc^r transductants from either the fast- or slow-growing class of transductants contained plasmids when analyzed by a small-scale plasmid preparation technique (see above). A second possibility is that P4DO102 contains sequences that are homologous to *K. pneumoniae* sequences and that P4DO102 integrates into the host chromosome through homologous recombination. This also seems unlikely due to the *recA* mutation present in hosts such as KP5614. A third plausible explanation is that contaminating P2 in our P4DO102 phage stock mediated site-specific recombination. This may have been accomplished by the formation of P2–P4 hybrid molecules through linkage of mutually homologous staggered cohesive ends (33) and subsequent P2 site-specific recombination into the P2 chromosomal *att* site. This possibility also seems unlikely, however, due to the low frequency of viable P2 phage present in our P4DO102 phage stock. When titers were determined, the P2-to-P4 ratio on C-1792 and DO25, respectively, was ca. 10^{-6}. Nevertheless, we further examined the possibility of contaminating P2 by coinfecting P4DO102 with P2 *A*am127, a DNA replication-defective (and hence lysis-defective), but integration-proficient, mutant. We expected a substantial increase in the efficiency of P4DO102 transduction if P2 *A*am127 could mediate P4 integration. Our results, however, showed no significant differ-

ence when P2 Aam127 was coinfected with P4DO102 (Table 2).

In our estimation, the most reasonable explanation for the ability of P4DO102 to transduce *K. pneumoniae* without a helper is that site-specific integration of phage P4 in *K. pneumoniae* does not require the P4-encoded *int* gene. In other words, P4DO102 may be naturally integration proficient in *K. pneumoniae*. At first, this explanation appears to be contrary to the data in our previous report (21). However, it is conceivable that our previous attempts to detect integrated P4 molecules in a lysogenic *K. pneumoniae* culture failed because the overwhelming majority of cells were lysogenized via plasmid formation and that the number of integrated plasmids was below our detection limits. This latter interpretation is consistent with the fact that only 0.1% of a P4DO102-infected culture acquires a Tc^r phenotype.

To distinguish among these various possibilities for the helper-independent transducing ability of P4DO102, we examined the genomes of both the fast- and slow-growing classes of *K. pneumoniae* Tc^r transductants with the DNA hybridization method described by Southern (28). Genomic DNA was prepared from five fast-growing and from five slow-growing clones. As controls, we used cultures of uninfected and P4DO100 (replication-proficient) lysogenic clones of KP5614. When the undigested genomic DNAs were subjected to electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled P4DO102 DNA, only KP5614(P4DO100) showed the presence of the faster-migrating plasmid DNA. In Fig. 2, we overexposed the film to demonstrate the absence of plasmids in all KP5614(P4DO102) transductants.

If P4DO102 had integrated into the chromosome, we expected to see junction fragments between P4DO102 and *K. pneumoniae* sequences after digestion of genomic DNA with the appropriate restriction enzyme(s). Figure 3 shows the hybridization of ^{32}P-labeled P4DO102 DNA to *Bam*HI-digested DNA. *Bam*HI cleaves a circular P4DO102 molecule into three fragments of 6.2, 2.2, and 0.9 kb. The 0.9-kb *Bam*HI fragment contains the *att* locus of P4. All of the slow-growing class and one of five of the fast-growing class lost the 0.9-kb fragment and gained two new fragments (X1 and X2) (Fig. 3). Surprisingly, four of five clones from the fast-growing class retained the 0.9-kb fragment, which appears to be in a twofold-lower stoichiometric ratio when compared with the 6.2- and 2.2-kb fragments. In addition, these clones also exhibited junction bands X1 and X2. In conjunction with the fact that free plasmids were not detectable in these lysogens, we conclude that these four clones contain two P4DO102 genomes integrated in a tandem arrangement. We suspect that the faster growth observed in the presence of tetracycline was due to a dosage effect of harboring two copies of the Tc^r gene.

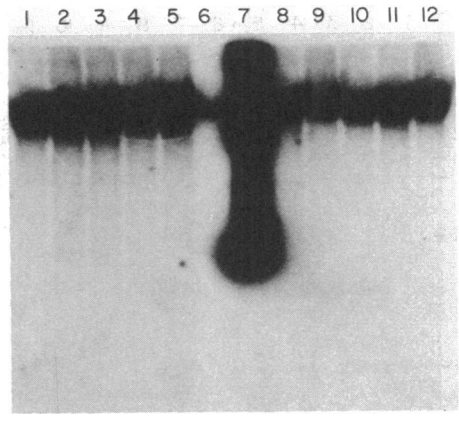

FIG. 2. Southern blot hybridization of undigested genomic DNA with ^{32}P-labeled P4DO102 DNA. Lanes 1 to 5, five independent clones of fast-growing (in the presence of tetracycline) KP5614(P4DO102) lysogens; lanes 8 to 12, five independent clones of slow-growing KP5614(P4DO102) lysogens; lane 6, KP5614; lane 7, KP5614(P4DO100). All lanes show hybridization to chromosomal DNA (slow-migrating form), but only lane 7 shows hybridization to plasmid DNA (fast-migrating form).

Although the 0.9-kb *Bam*HI fragment contains the P4 *att* locus, it also contains about 360 base

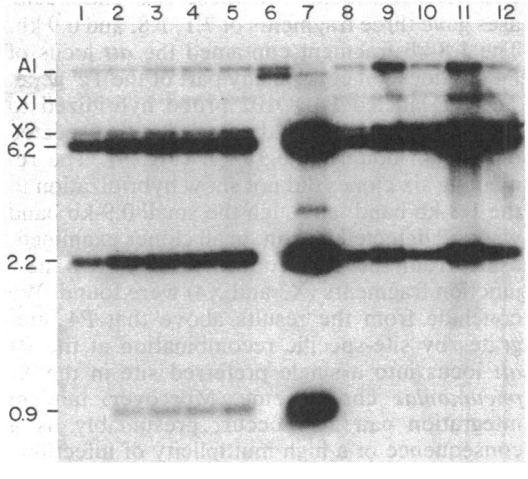

FIG. 3. Southern blot hybridization of *Bam*HI-digested genomic DNA with ^{32}P-labeled P4DO102 DNA. Lanes are as indicated in the legend to Fig. 2. P4DO102 fragments are the 6.2-, 2.2-, and 0.9-kb bands. X1 and X2 are junction fragments that resulted from site-specific integration. A1 is a fragment with homology to P4DO102 and may be derived from a P4-like cryptic prophage.

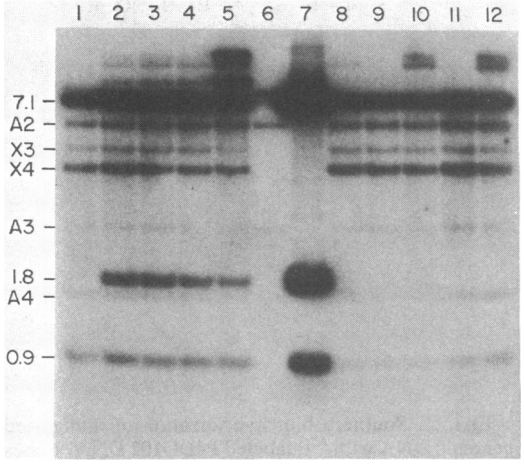

FIG. 4. Southern blot hybridization of genomic DNA doubly digested with *Eco*RI and *Bst*EII, using ^{32}P-labeled P4DO102 DNA as the probe. Lanes are as indicated in the legend to Fig. 2. P4DO102 fragments are the 7.1-, 1.8-, and 0.9-kb bands. X3 and X4 are junction fragments that resulted from site-specific integration. A2 to A4 are fragments with homology to P4DO102 and may be derived from a P4-like cryptic prophage.

pairs of the Tc^r gene. Thus, it is possible that the hybridization data of Fig. 2 and 3 may reflect integration at the Tc^r gene locus rather than P4 *att*-specific recombination. To rule out this possibility, genomic DNAs were digested with both *Eco*RI and *Bst*EII. Digestion of a circular P4DO102 molecule with these two endonucleases gave three fragments of 7.1, 1.8, and 0.9 kb. The 1.8-kb fragment contained the *att* locus of P4 but does not contain any part of the Tc^r gene. The ^{32}P-labeled P4DO102 probe hybridized to the 1.8-kb band only in the four clones suspected of tandem double integration (Fig. 4). The remaining six clones did not show hybridization to the 1.8-kb band, although the small 0.9-kb band was still detected. Again, in all clones examined, aside from the two control strains, two new junction fragments (X3 and X4) were found. We conclude from the results above that P4 integrates by site-specific recombination at the P4 *att* locus into a single preferred site in the *K. pneumoniae* chromosome. Moreover, tandem integration can also occur, presumably as a consequence of a high multiplicity of infection.

Use of P4 integrating vectors. As stated above, complementation analysis of certain *nif* loci with cloned DNA fragments has not been possible by using multicopy plasmids, due to the inhibitory effect of certain *nif* sequences (6, 23). We sought to overcome this difficulty by using P4DO102 to integrate a segment of cloned *nif* inhibitory sequences into the *K. pneumoniae* chromosome. One such segment of DNA is the *Eco*RI-*Bam*HI fragment which encodes the *nif* operons *nifLA* and *nifBQ* (23). We constructed a recombinant molecule (pDO201) by ligating together three gel-purified fragments: the *Eco*RI-*Bam*HI *nifLABQ* fragment, the *Eco*RI-*Bgl*II P4 *vir*1 αam52 *att*$^+$ fragment of P4DO102, and a *Bam*HI fragment derived from Tn*903* conferring kanamycin resistance (12); Fig. 1B plus C. Because the size of this molecule exceeds the packaging limit of a P4 capsid, pDO201 is incapable of P2-dependent lytic growth. Instead, it propagates solely as a plasmid in an Su$^+$ host. For this reason, we use the standard plasmid nomenclature (pDO201 rather than P4DO201) when referring to such derivatives.

We tested for integration of pDO201 in a *nifB* mutant strain (KP5617) and a *nifA* mutant strain (KP5611) of *K. pneumoniae*. Since pDO201 cannot grow as infective particles, pDO201 plasmid DNA was introduced into these Su$^-$ Rec$^-$ hosts by transformation. In general, low frequencies of Km^r transductants were recovered (30 to 70 clones per μg of DNA). The Km^r transductants were analyzed for their ability to complement *nifB* and *nifA* mutations in KP5617 and KP5611, respectively. All 48 KP5614(pDO201) and 18 KP5611(pDO201) lysogens examined were able to grow on nitrogen-free medium. Ten clones of each were further tested for the nitrogenase-catalyzed reduction of C_2H_2 and were found to contain wild-type (KP5614) levels of nitrogenase activity. We also examined the genomic DNA of these lysogens with hybridization strategy analogous to that described earlier. We used P4DO102 DNA as the probe instead of pDO201 DNA because the *nifALBQ* insert in pDO201 would hybridize to its chromosomal equivalent, as well as to the integrated form of pDO201. Genomic DNA from two clones of KP5611-(pDO201) and seven clones of KP5617(pDO201) was digested with endonuclease *Sma*I. *Sma*I cleaves a circular pDO201 molecule into four fragments of 6.8, 3.5, 3.1, and 2.9 kb. Only the 6.8- and the 3.1-kb fragments have homology to P4DO102. The 3.1-kb fragment contains the *att* locus of P4. In all nine lysogens examined (Fig. 5), the 3.1-kb fragment was missing, and two new bands appeared as presumptive junction fragments (Y1 and Y2). We conclude that pDO201 integrates site specifically into the Su$^-$ Rec$^-$ *K. pneumoniae* chromosome and complements the *nifB* and *nifA* mutations through merodiploid formation.

A second segment of *K. pneumoniae nif* DNA that has *nif* inhibitory properties in a multicopy state is the *nifH* promoter. V. Sundaresan of our laboratory transferred an *Eco*RI-to-*Sal*I fragment encoding a *nifH*::*lacZ* translational fusion of the *nifH* promoter to the *E. coli lacZ* gene

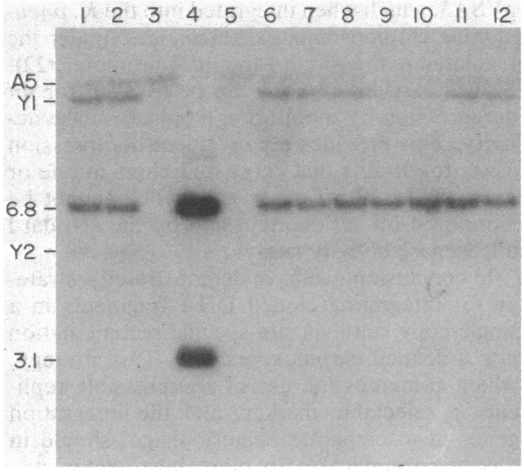

FIG. 5. Southern blot hybridization of *Sma*I (*Xma*I)-digested genomic DNA with ^{32}P-labeled P4DO102 DNA. Lanes 1 and 2, Independent clones of KP5611(pDO201); lanes 6 to 12, independent clones of KP5617(pDO201); lanes 3 and 5, KP5611 and KP5617, respectively; lane 4, *Sma*I-digested pDO201 plasmid DNA. pDO201 fragments with homology to probe DNA are the 6.8- and 3.1-kb bands. Y1 and Y2 are junction fragments that resulted from site-specific integration. A5 is a fragment with homology to P4DO102 and may be derived from a P4-like cryptic prophage.

from pVSA2 (a pBR322 derivative) to P4DO105 (see below) (30). The resultant P4 recombinant molecule, pVSA3 (Fig. 1E plus F), was used to transform *K. pneumoniae* KP5614 and UN4102 to Km^r. pVSA3 transformants of KP5614 and UN4102 did not inhibit nitrogen fixation, in contrast to the same fusion on the multicopy plasmid pVSA2 (V. Sundaresan, personal communication). To confirm that pVSA3 integrated into the *K. pneumoniae* chromosome, we examined five clones of KP5614(pVSA3) and one clone of UN4102(pVSA3) by using the DNA hybridization strategy described earlier. *Sma*I cleaves plasmid pVSA3 into a 10.7-kb fragment and a 6.8-kb fragment. When the *Sma*I-digested KP5614(pVSA3) andUN4102(pVSA3) genomes were probed with P4DO102 DNA, which has homology to both *Sma*I fragments of pVSA3, we found that the expected *att*-containing 10.7-kb band was missing and was replaced by two junction bands (Z1 and Z2). We conclude that pVSA3 also integrates site specifically. When expression of the *nifH-lacZ* fusion in KP5614(pVSA3) was examined by assaying for β-galactosidase activity, we found that the *nifH* promoter was active only under conditions of limiting O_2 and fixed nitrogen (22).

Derivative integrating vectors P4DO104 and P4D105. The success obtained with pDO201 prompted us to construct derivative vectors of P4DO102 with a variety of antibiotic resistance genes and unique cloning sites. P4DO104 is a Km^r derivative with a unique site for endonucleases *Eco*RI, *Pst*I, and *Bgl*II (Fig. 1D). *Xho*I is also a unique site but is located within the Km^r gene. P4DO105 has both the Tc^r gene of P4DO102 and the Km^r gene of P4DO104 (Fig. 1E). Unique endonuclease sites on P4DO105 are *Eco*RI, *Bal*I, *Sal*I (located within the Tc^r gene), and *Xho*I (located within the Km^r gene). Specific details of the construction of these vectors are described above.

DISCUSSION

We have shown that integration of P4 recombinant molecules containing an α amber mutation is due to a site-specific recombination event between the *att* locus of P4 and a single chromosomal *att* site in *K. pneumoniae*. Approximately 10% of the transductants contained two tandemly integrated P4 genomes, presumably as a result of a high multiplicity of infection of packaged P4 particles. In contrast, 15 of the integrated P4 genomes examined which had been introduced into *K. pneumoniae* by transformation showed integration of a single molecule, although $CaCl_2$-treated competent cells are capable of cotransformation by multiple plasmid molecules (3). One possibility may be that the double integrated lysogens arose as a result of dimer formation before integration rather than as a result of two independent integration events. Since phage DNA enters the host in a linear form, it is possible that dimers (or multimers) can be formed by the annealing of cohesive ends between coinfecting P4 chromosomes. In contrast, dimerization of circular plasmid monomers is usually by *recA*-dependent homologous recombination (2).

In contrast to the case found in *E. coli* (8), phage P4 site-specific integration in *K. pneumoniae* apparently does not require the phage-encoded *int* gene. It is possible that *K. pneumoniae* may have a host function that is analogous to the P4 *int* function. An alterantive possibility is that *K. pneumoniae* M5al and its derivatives contain a P4-like cryptic prophage which provides a P4-like *int* product in trans. Although we have made no attempt to distinguish between these possibilities, we did observe, in all of our Southern blot hybridizations of ^{32}P-labeled P4DO102 DNA to the *K. pneumoniae* genome, faintly hybridizing host-specific fragments which were unaltered in size upon phage P4 integration (Fig. 3, A1; Fig. 4, A2, A3, and A4; Fig. 5, A5; Fig. 6, A6). Because these bands did not change in size after integration, they are probably not partially homologous chromosomal *att* sites, but could be the remnants of a P4-like cryptic prophage chromosome.

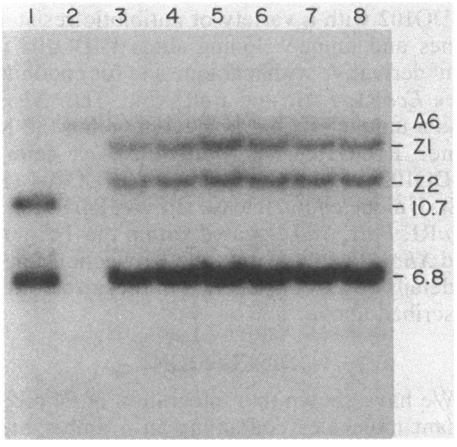

FIG. 6. Southern blot hybridization of *Sma*I (*Xma*I)-digested genomic DNA with ^{32}P-labeled P4DO102 DNA. Lanes 3 to 7, Independent clones of KP5614(pVSA3); lane 8, UN4102(pVSA3); lane 2, KP5614; lane 1, *Sma*I-treated pVSA3 plasmid DNA. pVSA3 fragments with homology to probe DNA are the 10.7- and 6.8-kb bands. Z1 and Z2 are junction fragments that resulted from site-specific integration. A6 is a fragment with homology to P4DO102 and may be derived from a P4-like cryptic prophage.

Our results with P4 integrating vectors suggest that cloned DNA fragments can be integrated in a single-copy state for physiological analysis. The vector most often used for merodiploid constructions of this type is phage λ. Merodiploids are constructed by either λ site-specific recombination or *rec*A-dependent homologous recombination. Although λ vectors are useful in *E. coli*, they are not readily adaptable to *K. pneumoniae* due to the natural host range resistance of this enteric species to phage λ infection. The integrating phage P4 vector is thus an attractive alternative. In a comparison of the P4 and λ systems, the unique feature of phage P4 is that recombinant P4 molecules can replicate as a plasmid.

We observed that the frequency of site-specific integration by P4DO102 varied considerably among several *E. coli* strains that we examined. If differences in host background are the cause of this observed difference in the integration efficiency, then perhaps the best strategy to construct merodiploids would be to integrate P4 derivatives into an F′ (or R′) plasmid that carries the P4 chromosomal *att* locus (near 96.3 min on the *E. coli* chromosome) in a host such as CK111. Subsequently, the lysogenic plasmid could be mated into other recipients.

The integration of P4 derivatives is currently being employed in the engineering of stable strains for use in protein purification (P. McLean, F. Hanson, and W. Orme-Johnson, personal communication). An example is pVSA3, which when integrated into the *K. pneumoniae* chromosome, expresses *lacZ* under the regulated expression of the *nifH* promoter (22). Strains carrying pVSA3 can be assayed for *nif* derepression by monitoring β-galactosidase activity. This provides a convenient derepression assay for strains that carry mutations in one or more of the 17 *nif* genes and hence cannot be monitored for *nif* derepression by the standard nitrogenase activity assays.

In conclusion, we have demonstrated a strategy for integrating cloned DNA fragments in a single-copy state via site-specific recombination into a defined chromosomal site. This strategy, which combines the use of a supressible replicon, a selectable marker, and the integration genes of a temperate bacteriophage, should in theory be applicable to other host-vector systems.

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